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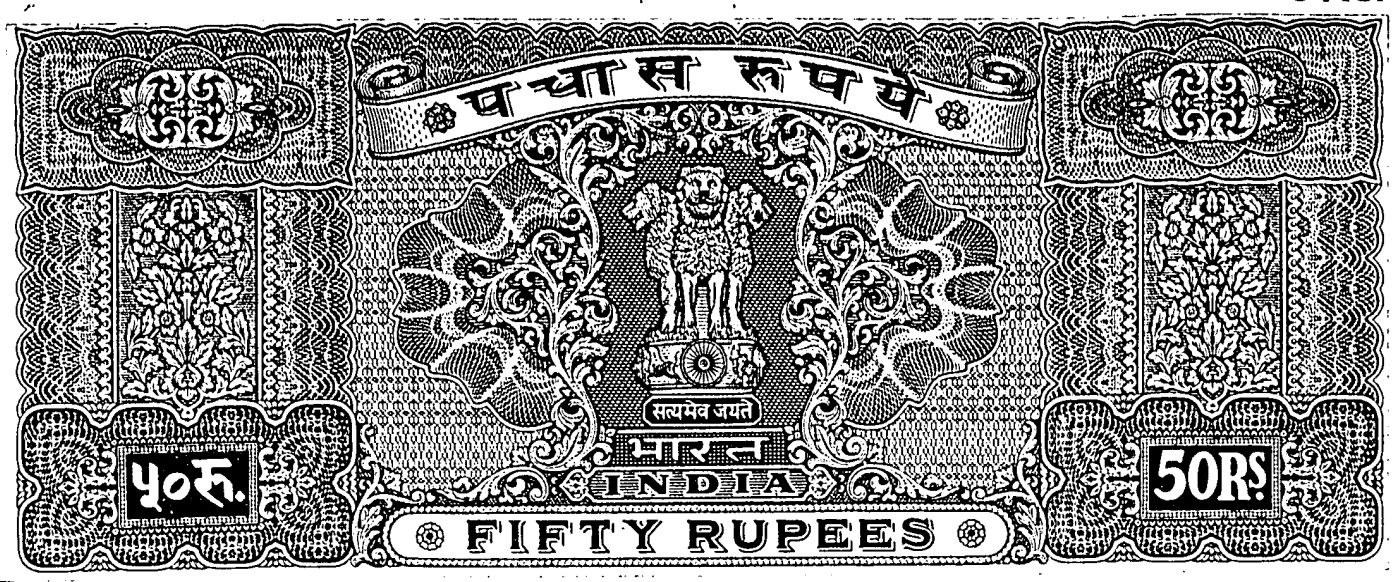




**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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|---|---|---|
| Application No. | : 09/811,766 | Confirmation No. 9295 |
| Applicants | : Appu Rao *et al.* | |
| Filed | : March 19, 2001 | |
| TC/AU | : 1654 | |
| Examiner | : Michael V. Meller | |
| Docket No. | : 000132850-0007 (former no. 46598-00007) | |

**Mail Stop Amendment**
**Commissioner for Patents**
**P.O. Box 1450**
**Alexandria, VA 22313-1450**

# DECLARATION
# (37 CFR 1.132)

The undersigned declares as follows:

(1) I am one of the applicants in the above-identified patent application.

(2) I have read and am familiar with the specification and claims of the instant application.

(3) I have read and am familiar with the Examiner's reasons for rejection of the claims of the present application and am also familiar with the prior art cited during the course of prosecution of the instant application.

(4) The references cited by the Examiner can be categorized into 2 categories:

Category 1: References teaching use of proteolytic enzymes for hydrolyzing the soy flour protein.

Category 2: References teaching use of Papain for hydrolyzing the soy flour protein.

(5) The references included in Category 1 are EP 0 148,600 (Cipollo et al.), Thomas et al., Chigurupati et al., Olsen et al., Daboge et al. and Schoenmaker et al.

(6) The reference included in Category 2 is Satoh et al.

(7) The references categorized in Category 1 (taken individually or in combination) teach the use of proteolytic enzymes for hydrolyzing the soy flour protein and the reference categorized in Category 2 teaches the use of papain for hydrolyzing the soy flour protein.

(8) The Examiner stated that a person skilled in the art would be motivated to combine the teachings of the references category in Category 1 with the reference (either individually or in combination) in Category 2 and would arrive at the presently claimed invention.

(9) I respectfully submit that the teachings of the references in Category 1 can be combined with the teachings of the reference cited in Category 2 in five different ways, each of which is briefly outlined here below:

**Option 1:** "X" amount of proteolytic enzyme could be mixed with "Y" amount of papain to obtain a protein hydrolyzing enzyme composition and such protein hydrolyzing enzyme composition could be added to the aqueous slurry of defatted soy flour to obtain the protein hydrolysate. The temperature and pH value at which the enzymatic composition will be added to the aqueous slurry of defatted soy flour, the time period for which the enzymatic reaction

should proceed etc. are to be determined practically. Also, the values of "X" and "Y" are to be determined to obtain the products with the desired characteristics.

Together with other co-inventors I conducted experiments before the filing of the instant application to identify the suitability of the basic process mentioned above. A flow chart depicting one of such trials is shown below:

preparing an aqueous slurry of DSF

mixing both the enzymes in equal proportions

subjecting the slurry to hydrolyzation with the above enzyme composition at a pH 7.0 and at a temperature of 43°C for 3 ½ hrs

inactivating by increasing the temperature above 70°C

separating solids

drying

protein hydrolysate

**Brief Description of the Process and the Results Obtained:**

Both enzymes, fungal protease and papain, were mixed in equal proportions to obtain a protein hydrolyzing enzyme composition. The protein hydrolyzing enzyme composition thus obtained was added to a slurry of DSF and the hydrolysis was carried out at a pH 7.0 for 3 ½ h at a temperature of 43°C at 1% concentration. The hydrolyzed liquor was spray dried and evaluated for degree of hydrolysis (DH) and nitrogen content. The DH and nitrogen content was 18.1 - 19% and 9-9.2% respectively.

Together with other co-inventors I noticed the following difficulties in the above-mentioned basic process:

a. It was noticed that it was not possible to keep both the enzymes activated to perform the hydrolysis. More particularly, it was noticed that the papain was in activated stage when the pH value was in the range of 6.0 to 7.0 and when the temperature is maintained at 55°C. On the other hand, it was observed that fungal protease was in

activated state when the pH value was in the range of 7.8 to 8.0 and when the temperature is maintained at 43°C. Thus, it was noticed that when papain was in the activate stage, the fungal protease was not in the activated stage and vice versa. This affected the degree of hydrolysis of the final product.

**b.** Even increasing the enzyme concentration to 2% did not improve the degree of hydrolysis and nitrogen content of the product. The optimum temperature for maximum activity for papain is 55°C and 43°C for protease.

**c.** It was further noticed that at 55°C, fungal protease used to get partially deactivated, which is yet another reason for lesser degree of hydrolysis and nitrogen content of the product. This experiment showed that the degree of hydrolysis of the final product was lower with DH 18.1-19% and nitrogen content was 9-9.2%.

**d.** Thus, it was not possible to obtain the desired objectives by using the above-mentioned process.

**Option 2:** To the aqueous slurry of defatted soy flour Papain could be added to start with under particular temperature and pH values and the hydrolysis of the soy flour slurry would be allowed to proceed for a particular time period. Thereafter, the papain present in the reaction mixture would be de-activated in a known manner to obtain an intermediate product. Subsequently, proteolytic enzyme would be added to the intermediate product thus obtained under particular temperature and pH values and the further hydrolysis would be allowed to proceed for a particular time period. The proteolytic enzyme would then be deactivated to obtain the final product.

Together with other co-inventors I conducted experiments before the filing of the application to find the suitability of the basic process mentioned above. A flow chart depicting one of such trials is shown below:

Preparing an aqueous slurry of DSF

subjecting the slurry to hydrolysis with the enzyme papain at a pH 7.0 and at a temperature of 55°C for 2hrs

inactivation by heating at temperature 80°C

at this stage fungal protease is added and hydrolysis is continued for 2 hrs

inactivation by heating to 73°C

separating solids

drying

protein hydrolysate

**Brief description of the Process and the Result obtained:**

An aqueous slurry of DSF was prepared and was subjected to hydrolysis with the enzyme papain at a pH 7.0 at a temperature of 55°C for 2 hrs. Then the enzyme was inactivated by heating to a temperature of 80°C. Then fungal protease was added and hydrolysis is continued for 2 hrs. Then the reaction was inactivated and the hydrolyzed liquor was spray dried and evaluated for degree of hydrolysis (DH) and nitrogen content. The DH and nitrogen content was 22-23% and 9-9.8% respectively.

Together with other co-inventors I noticed the following difficulties in the above-mentioned basic process:

a. It was observed that Papain needed higher temperature for inactivation, i.e., 80°C. Protease was rendered inactive in the temperature range of 72-75°C. Though both enzymes are not specific, the action of both the enzymes towards the peptide bonds is different.
b. When papain was added first, there was a considerable loss of nitrogen during the precipitation of larger size peptides, during first 2 hrs of hydrolysis.
c. Also the product was bitter. This experiment showed that the degree of hydrolysis of the final product was lower with DH 22-23% and nitrogen content was 9-9.8%.

d. Thus, it was not possible to obtain the desired objectives by using the above-mentioned process.

**Option 3:** To the aqueous slurry of defatted soy flour Papain is added to start with under particular temperature and pH values and the hydrolysis of the soy flour slurry would be allowed to proceed for a particular time period to form an intermediate product in the slurry. Subsequently, proteolytic enzyme would be added to the slurry without stopping the reaction under particular temperature and pH values and the further hydrolysis would be allowed to proceed for a particular time period. The enzymes present in the reaction mixture would then be deactivated to obtain the final product. This is a single stage or, alternatively, a single pot process and papain enzyme is not deactivated at an intermediate stage.

Together with other co-inventors I conducted experiments before the filing of the application to find the suitability of the basic process mentioned above. A flow chart depicting one of such trials is shown below:

Preparing an aqueous slurry of DSF

subjecting the slurry to hydrolysis with the enzyme papain at a pH 7.0 and at a temperature of 55°C for 2 hrs

lowering the temperature to 43°C

addition of protease at this stage

continuation of hydrolysis for 1 ½ hrs

inactivation

separating solids

drying

protein hydrolysate

**Brief description of the Process and the Result obtained:**

Together with other co-inventors I noticed the following difficulties in the above-mentioned basic process.

a. The product of this process did not give high degree of hydrolysis.
b. The addition of papain first generated bitter peptides as its mode of action is different.
c. Papain generated peptides of relatively high molecular weight which acted as inhibitors of fungal protease.
d. Also, if papain is not inactivated after hydrolysis for 2 hrs in the first step the enzyme can also contribute to the inactivation of fungal protease added in the second step of the reaction. (Papain and fungal protease are also proteins and can act against each other.) The experiment carried out in this manner showed the degree of hydrolysis of the final product in the range 14-16%. The nitrogen content of the product was 9-9.6%.
e. This process resulted in bitter peptides. Hence, adding papain first did not give a hydrolysate with high degree of hydrolysis and high nitrogen content like in the present invention.

**Option 4:** To the aqueous slurry of defatted soy flour proteolytic enzyme is added to start with under particular temperature and pH values and the hydrolysis of the soy flour slurry is allowed to proceed for a particular time period. Thereafter, the proteolytic enzyme present in the reaction mixture is de-activated in a known manner to obtain an intermediate product. Subsequently, papain is added to the intermediate product thus obtained under particular temperature and pH values and further hydrolysis is allowed to proceed for a particular time period. The papain would then be deactivated to obtain the final product.

Together with other co-inventors I conducted experiments before the filing of the application to find the suitability of the basic process mentioned above. A flow chart depicting one of such trials is shown below:

Preparing an aqueous slurry of DSF

subjecting the slurry to hydrolysis with the enzyme protease at a temperature 70-75°C and at pH 7.0 for 2 hrs

inactivation

lowering the temperature to 50-55°

addition of papain

continuation of hydrolysis for 1 ½ hrs

inactivation

separating solids

drying

protein hydrolysate

**Brief description of the Process and the Result obtained:**

An aqueous slurry of DSF was prepared and was subjected to hydrolysis with the enzyme fungal protease at a pH 7.0 at a temperature of 70-75°C for 2 hrs. Then it was inactivated. Subsequently papain was added at 50-55°C, 1 ½ hrs, and hydrolysis is continued for 1 ½ hrs. Then the reaction was inactivated and the hydrolyzed liquor was spray dried and evaluated for degree of hydrolysis (DH) and nitrogen content. The DH and nitrogen content was 30-35% and nitrogen content was not determinable.

Together with other co-inventors I noticed the following difficulties in the above-mentioned basic process.

a. Inactivation of fungal protease required a temperature of 70-75°C. Optimum temperature for papain activity is 50-55°C. Hence, before addition of papain the temperature had to be brought in the range 50-55°C in order to get maximum efficiency with respect to hydrolysis.
b. Also raising the temperature after hydrolysis with fungal protease in the first step lead to partial precipitation of some larger peptides. This lead to loss of nitrogen content in the final product. Though relatively the

degree of hydrolysis is in the range 30-35%, the nitrogen content of the product is low which was found to be undeterminable.

c. Hence the product prepared in this route resulted in low nitrogen recovery.

**Option 5:** To the aqueous slurry of defatted soy flour proteolytic enzyme is added to start with under particular temperature and pH values and the hydrolysis of the soy flour slurry is allowed to proceed for a particular time period to form an intermediate product in the slurry. Subsequently, papain is added to the slurry without stopping the reaction under particular temperature and pH values and the further hydrolysis would be allowed to proceed for a particular time period. The enzymes present in the reaction mixture would then be deactivated to obtain the final product. Please note that this is a single stage or otherwise a single pot process and proteolytic enzyme is not deactivated at an intermediate stage.

*This approach includes adding the protease first (according to an optimum temperature/pH/time), the papain is then added (according to an optimum temperature/pH/time), and both enzymes are inactivated. The desired product results, all according to the present invention.*

The product prepared according to this methodology after hydrolysis with fungal protease (2 hrs) at 43°C followed by papain and inactivation lead to a product with higher degree of hydrolysis and higher nitrogen content. Inactivation of both enzymes by heating at the end of hydrolysis is necessary because at the end of 3 ½ hrs the degree of hydrolysis of the hydrolysate is very high. Hence, the peptide size was low and there was no loss of peptides due to precipitation in the present invention. Thus, only by the procedure of the present invention is it possible to obtain a protein hydrolysate that is soluble at all pH, non-bitter, 95-98% nitrogen solubility index and 35-45% degree of hydrolysis. The product is free from lipoxygenase or urease activity and has a similar amino acid make up as the starting matter.

(10) Together with other co-inventors I was able to generate the following comparative chart which compares the characteristics of the products prepared in the processes of Options 1 through 4 as set forth above with the product of the invention as claimed:

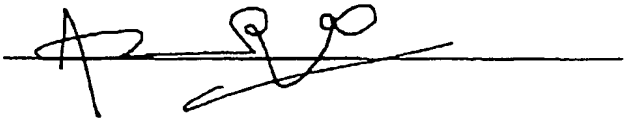
| CHARACTERISTICS | 1 | 2 | 3 | 4 | PRODUCT OF THE PRESENT INVENTION |
|---|---|---|---|---|---|
| Degree of Hydrolysis | 18.1-19% | 22-23% | 14-16% | 30-35% | 35-45% |
| Nitrogen content | 9-9.2 % | 9-9.8% | 9-9.6% | Not determinable | 10.5-11% |
| Threshold reception of Bitterness | Not perceptible | Perceptible | Perceptible | Not perceptible | Not perceptible |
| | | | | | |

(11) I respectfully state that one skilled in the art would be tempted to adopt the process described in the first option, i.e., obtaining a protein hydrolyzing enzyme composition comprising "X" amount of proteolytic and "Y" amount of papain and using the same for hydrolysis because of its ease in application. Thereafter, a person skilled in the art would be tempted to try out the process described in Options 2 or 4 because they of their ease in industrial implementation. Only as their last option would such a person opt for the processes described in Options 3 and 5 because of the difficulty in adopting such processes as an industrial process.

(12) I further respectfully state that it is not technically possible for the teachings of the cited references to be combined in any way that would result in the product produced by the process as claimed in the present invention.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 an that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant/Inventor name: Appu Rao Gopala Rao Appu Rao

Declarant/Inventor signature: 

Date: Feb 11, 2005